Enzyme-Linked Immunosorbent Assay for Detection of *Thielaviopsis basicola*

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ABSTRACT

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Field isolates of *Thielaviopsis basicola*, the causal agent of black root rot of cotton (*Gossypium hirsutum*), were grown in Czapek-Dox broth amended with dialyzed carrot extract. Soluble protein extracts of chlamydospores and mycelium were used to raise polyclonal mouse ascites antibodies. The immunoglobulin G antibody fraction was purified and biotin-labeled to devise a fungal capture sandwich enzyme-linked immunosorbent assay (ELISA). ELISA detected both brown and gray cultural types of *T. basicola* and had negligible cross-reactivity with other soilborne fungi commonly found in the San Joaquin Valley of California cotton field soils. The minimum detection limit of ELISA was between 1 and 20 ng of *T. basicola* protein depending on the assay. *T. basicola* could be detected in cotton roots 2 days after inoculation. At this time, initial symptoms were apparent. The antibody also was used to observe *T. basicola* on cotton roots with immunofluorescence microscopy.

Black root rot of cotton (*Gossypium hirsutum* L.), caused by *Thielaviopsis basicola* (Berk. & Broome) Ferraris (synanamorph *Chalara elegans* Nag Raj & Kendrick), is a disease of increasing importance in the San Joaquin Valley of California (B. A. Roberts, Kings County Farm advisor, *personal communication*). *T. basicola* is a common soil inhabitant in both cultivated and noncultivated soils (42) that causes characteristic black necrotic lesions on the main and lateral roots of over 137 plant species (15,21). *T. basicola* is most damaging to the cotton plant during the early stages of development, entering the plant through root hairs and then successively invading all root tissues (11,26). Histological studies of root-infecting fungi are difficult because of the presence of diverse fungal colonies found on roots. Hyphae of most fungi appear similar when visualized with conventional histological stains, making the identification of individual species, such as *T. basicola*, difficult.

A number of researchers have reported on the production of antisera with varying degrees of specificity for plant-pathogenic fungi (9,30,41). Fluorescent antibody techniques also have been used in attempts to distinguish particular fungal pathogens growing in host tissue (16,24,27,32). Problems encountered with these approaches include a lack of specificity of the antisera, the autofluorescence of tissue, and the requirement of special fluorescence detection equipment. Enzyme-linked immunosorbent assays (ELISA) have found widespread application in plant pathology and are routinely used for detection and identification purposes (12,13,23,37). Their applicability to the detection of fungal pathogens in host tissues has been reported (1,28,39). Gerik et al (17) combined immunochemical and histochemical techniques and developed an immunoenzymatic staining procedure to visualize *Verticillium dahliae* hyphae in plant tissue by deposition of insoluble dye. The dye formed a colored precipitate outlining the fungal hyphae that were observed on and in the root cortex of the host with a dissecting microscope.

Several researchers have quantified fungi in plant tissue with double-antibody sandwich (DAS)-ELISA (7,14,29,37). DAS-ELISA could be especially useful for detecting antigens in complex mixtures such as soil or plant extracts, because the bound antibody specifically captures the antigen(s) of interest, while irrele-

vant material is removed in the initial wash step. DAS-ELISA usually requires two antibodies produced in different animals and anti-immunoglobulin antibodies conjugated to an enzyme. Our objective was to determine whether *T. basicola* could be detected serologically in plant tissue with immunofluorescence microscopy and a fungal capture sandwich ELISA with biotinylated and nonbiotinylated polyclonal ascites antibodies raised in mice against a soluble fraction of the fungus. An immunochemical method by which *T. basicola* could be specifically identified, quantified, and observed directly, either as hyphae growing externally or internally on cotton roots or as chlamydospores in the soil, would facilitate epidemiological studies of black root rot. A preliminary report describing a portion of this work has been published (19).

MATERIALS AND METHODS

Antigen preparation. Isolates of *T. basicola* (Table 1) were retrieved from San Joaquin Valley cotton soil by the carrot disk method described by Yarwood (42). The fungus was obtained in pure culture on potato-dextrose agar (PDA) and maintained by single-spore transfers. Flasks containing 250 ml of carrot dialysate (1.0 g of carrot extract per liter of H_2O), Czapek-Dox broth (35 g/L), and asparagine (2 g/L) were inoculated with 100 μl of spore suspension (approximately 10^4 spores per milliliter) of three brown (isolates 24, 28, and 33; Table 1) and three gray (isolates 22, 26, and 30; Table 1) isolates of *T. basicola*. Cultures were incubated at room temperature on a rotary shaker for 2 wk. Mycelia were harvested by filtering through four layers of cheesecloth and were washed two times in several volumes of phosphate buffered saline (PBS; 0.14 M NaCl, 0.0025 M $NaH_2PO_4 \cdot 2H_2O$, and 0.0075 M Na_2HPO_4, pH 7.4). The fungal tissue was frozen with liquid nitrogen and ground with a mortar and pestle. An equal volume of PBS was added to the ground fungal material, and the suspension was sonicated eight times, 30 s each time, in an ice bath with a Branson Sonifier cell disruptor 185 (Branson Sonic Power, Smith/Klein Company, Danbury, CT) at maximum power. The suspension was centrifuged at 12,000 *g* for 10 min in a Sorvall SS-3 centrifuge (Sorvall Instruments Division, Du Pont Company, Willmington, DE). The cytosol supernatant was removed and stored at −80 C until needed. The pellet, containing the cell wall fragments, was resuspended in a minimal volume of PBS and stored separately at −80 C. The protein concentration for the cytosol supernatant was determined by the method of Bradford (6). The concentration for the cell wall suspension was determined by dry-weight measurements. Other soilborne fungi (isolates 33–40; Table 1), commonly collected from cotton roots and soil from San Joaquin Valley cotton fields, were prepared in a similar manner.

TABLE 1. Isolates of fungi from California included in this study

Fungus (cultural type) Isolate	Substrate and origin	Source[a]
Thielaviopsis basicola (brown)		
1, 12, 14–16, 18, 19, 24, 25, 28, 29, 33	Cotton soil, Kings Co.	1
T. basicola (gray)		
2–11, 20–23, 26, 27, 30–32	Cotton soil, Kings Co.	1
T. basicola (—)[b]		
13, 17	Cotton soil, Kings Co.	1
Rhizoctonia solani		
34	Cotton roots, Kern Co.	2
Fusarium oxysporum f. sp. *vasinfectum*		
35	Cotton roots, Fresno Co.	3
Verticillium dahliae		
36	Pepper plant, Monterey Co.	1
V. dahliae		
37	Cotton roots, Kings Co.	2
Sclerotinia sclerotiorum		
38	Cotton soil, Kings Co.	1
S. minor		
39	Cotton soil, Kings Co.	1
Trichoderma sp.		
40	Cotton soil, Kings Co.	1
Penicillium sp.		
41	Cotton soil, Kings Co.	1

[a] 1 = B. A. Holtz, Univ. Calif., Berkeley; 2 = A. R. Weinhold, Univ. Calif., Berkeley; 3 = J. G. Hancock, Univ. Calif., Berkeley.
[b] Cultural type is unknown.

Immunization of mice. Six Swiss Webster mice were immunized with separate cytosol-protein suspensions, and six mice were immunized with separate cell wall preparations of the *T. basicola* isolates described above. Mice were immunized with one priming dose and two booster doses 7 and 21 days after the priming dose. Each dose consisted of 100 μg of antigen in approximately 0.15 ml of PBS, emulsified with one mouse dose of Ribi adjuvant (MPL plus TDM emulsion, Ribi Immunochem Research, Hamilton, MT), and distributed subcutaneously in two to four sites on the back (approximately 0.05 ml per site). Blood was taken from the tail vein 5–10 days after the second booster dose. The blood was allowed to clot for 15 min at room temperature and 15 min on ice, the sera were separated by centrifugation (12,000 *g* for 10 min at 4 C), and aliquots were stored at −80 C. The sera were tested by ELISA and western blot analysis. Mice that responded best were used to produce polyclonal ascites fluid (22).

Production of polyclonal ascites. Mice that developed a high-titer response to *T. basicola* antigens received an additional booster dose. Three days later they were given an intraperitoneal injection of 10^6–10^7 T-180 sarcoma cells (American Type Culture Collection, Rockville, MD, [ATCC] TIB66) in 1 ml of physiological saline. From the seventh day after the cells were injected, the mice were monitored daily for ascites development, and ascites fluid was harvested by peritoneal tap with an 18-gauge needle. Most mice produced from 5 to 20 ml of antibody-containing ascitic fluid over a 1-wk period. The mice were euthanized as soon as they showed any sign of debility or distress.

Testing of serum. Indirect ELISA was used to titer polyclonal ascites antibodies (35,40) and to test their specificity for brown and gray isolates of *T. basicola* and other fungi commonly found in cotton field soil. ELISAs were done in microtiter plates (Immulon II, Dynatech Corp., Alexandria, VA). The plates were coated with soluble protein suspension, diluted with standard ELISA coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, and 0.003 M NaN_3, pH 9.6) so that each well received 1 μg of antigen in 0.1 ml. The coated plates were incubated at 4 C for 16 h and washed three times with PBS-Tween (0.01 M $KH_2PO_4 \cdot K_2HPO_4$, 0.15 M NaCl, 0.02% NaN_3, and 0.05% Tween 20, pH 7.4) from a squeeze bottle and dried by rapping on lint-free towels. The plates were tightly covered during all incubation steps to prevent evaporation. Blocking buffer (PBS-Tween containing 1% bovine serum albumin [BSA]), used to prevent adventitious binding, was filtered (0.45 μm) to remove aggregates, added to the plates at 0.1 ml per well, and allowed to incubate at room temperature for a minimum of 30 min. The blocking buffer was discarded, and the *T. basicola* antibody, diluted with blocking buffer, was added to the plates at 0.1 ml per well. The antibody was allowed to incubate at room temperature for 2 h. The plates were washed three times with PBS-Tween. Alkaline phosphatase-conjugated goat–anti-mouse immunoglobulin G (IgG) conjugate, diluted 1:1,000 with blocking buffer, was added to the plates at 0.1 ml per well for 2 h at room temperature. The wells were again washed with PBS-Tween, and 0.1 ml of substrate solution (*p*-nitrophenyl phosphate, Sigma 104 phosphatase substrate [Sigma Chemical Co., St. Louis]) was added at 1 mg/ml in 10% (w/v) diethanolamine-HCl buffer (DEA buffer; 0.4 mM $MgCl_2$, and 3 mM NaN_3, pH 9.8, stored at 4 C in the dark) warmed to room temperature.

The rate of color development was monitored on a Multiskan ELISA reader (Flow Laboratories, Inc., McLean, VA) interfaced with a Macintosh computer, and the rates of the reaction (ΔA_{405nm}/min $\times 10^3$) were calculated by linear regression (22,33, 35), usually from three or more readings at 5-min intervals, as

the change in absorbance or color development above the blank from the time the substrate was initially added. The rate of substrate color development is proportional to the amount of antigen present in the sample.

Biotinylation of antibodies. ImmunoPure G Immobilized Protein G columns (Pierce Chemical Co., Rockford, IL) were used for isolation and purification of *T. basicola*-specific IgG from mouse polyclonal ascites (3). For biotinylation, the purified IgG was dialyzed overnight against three changes of 0.1 M sodium carbonate-bicarbonate buffer, pH 9.0. Protein concentration was determined after dialysis and adjusted to approximately 1.0 mg/ml by dilution with bicarbonate buffer. One milligram of long-chain biotinyl-*N*-hydroxysuccinimide ester (Pierce) was dissolved immediately before use in 100 μl of dimethyl sulfoxide. The solution was brought to a concentration of approximately 1 mg/ml with bicarbonate buffer, and 120 μl was added to 1.0 ml of antibody and mixed immediately (2,18,31). The solution was placed on a rotary shaker for 2 h at room temperature and dialyzed overnight against three washes of PBS with 0.1% sodium azide to remove unconjugated biotin. The conjugate was stored at 4 C.

Fungal capture sandwich ELISA. Biotinylated polyclonal IgG was used in a fungal capture sandwich ELISA to detect and quantify *T. basicola* in plant tissue. Cotton seedlings, 1 day after emergence, were each inoculated with a 0.1-ml spore suspension (10^4 spores per milliliter) of *T. basicola* (isolate 26; Table 1) phialospores, which were placed as a drop on the hypocotyl. The phialospores (endoconidia) were harvested as described by Christias and Baker (10). The seedlings were transplanted in sand and grown in a 15-C greenhouse chamber. The hypocotyls were removed 0, 2, 8, 12, and 19 days after inoculation, and a 2.5-cm section of each hypocotyl was suspended in PBS and stored at −80 C until the completion of the experiment. Soluble cotton-root homogenates were prepared as described for the initial immunogen, and the fungal capture sandwich ELISA was used to detect *T. basicola*. The ELISA wells were coated with 0.1 ml of unmodified *T. basicola*-specific IgG (approximately 1 μg in coating buffer). The coated wells were incubated overnight at 4 C and washed three times with PBS-Tween. Blocking buffer (PBS-Tween containing 0.1% gelatin) was added, 0.1 ml per well, for 30 min at room temperature. After discarding the blocking buffer, 0.1 ml of soluble antigen extracts or cotton-root homogenates, diluted with blocking buffer, was added for 2 h at room temperature.

In this step, the antigen in the test sample is selectively trapped by the solid-phase *T. basicola*-specific antibody. The plates were washed three times with PBS-Tween, and 0.1 ml of biotin-labeled *T. basicola*-specific IgG, diluted 1:100 with blocking buffer, was added to each well for 2 h at room temperature. Control wells received blocking buffer instead of either antigen or biotinylated IgG. Nonbiotinylated *T. basicola*-specific IgG was used in place of biotinylated IgG as another control.

The plates were washed again, and 0.1 ml of extravidin alkaline phosphatase conjugate (Sigma), diluted 1:1,000 with blocking buffer, was added to each well for 1 h. After the wells were washed, substrate solution was added, and the rate of color development was recorded. The ELISA rates from the diseased root extracts were compared to ELISA rates from *T. basicola* standards, which were fitted by iterative regression to a classical four-parameter logistic equation (8,22,34) with Passage II (Passage Software, Inc., Fort Collins, CO) computer software. Quantification of *T. basicola* in extracts of diseased cotton roots was done by interpolation from the rearranged four-parameter logistic equation: $x = 10^R$, where $R = \log_{10}\{[(a_1 - a_2/y - a_2) - 1]/a_4\} + \log_{10}(a_3)$ and a_1 = lower asymptote, a_2 = upper asymptote, a_3 = midpoint of curve, and a_4 = slope.

Immunofluorescence microscopy. Immunofluorescence microscopy was used to visualize *T. basicola* on cotton roots. The roots were obtained from diseased seedlings sampled from fields in Kings County, CA. Buffer volumes used were variable and adjusted to always give complete submergence of the sample (32). Sections of infected cotton hypocotyls were placed in 1.5-ml Eppendorf centrifuge tubes and washed three times, 5 min each time, with phosphate buffer (0.1 M KH_2PO_4 and 0.01 M K_2HPO_4, pH 7). *T. basicola*-specific ascites IgG, diluted 1:100 with blocking buffer (PBS containing 1% BSA), was added to the roots for 1 h at 37 C. The roots were washed three times, 5 min each time, with blocking buffer, and four more times, 10 min each time, with PBS-Tween.

The roots were incubated for 1 h at 37 C with goat-anti-mouse IgG conjugated with tetramethylrhodamine isothiocyanate (Sigma), diluted 1:16 with blocking buffer. The roots were again washed four times, 10 min each time, with PBS-Tween. They were removed from the Eppendorf tubes, an epidermal peel was taken from each root and mounted on microscope slides with 2% *n*-propyl gallate in 70% glycerol-PBS, and examined under a fluorescence microscope (Nikon Optiphot compound microscope, Nikon, Inc., Instrument Group, Melville, NY; equipped with episcopic-fluorescence, a 330–388 nm interference excitation filter, and a 435 nm barrier filter). Photographs were made on Ektachrome 200 ASA film, with exposure times determined by an integrating photometer (Nikon Microflex UFX, Walter Kahune—Distributor, San Raphael, CA).

Denaturing gel electrophoresis and western blotting. Soluble proteins from *T. basicola* extracts were resolved by denaturation at 100 C in 0.1% sodium dodecyl sulfate (SDS) under reducing conditions and subjected to electrophoresis in a 10% polyacrylamide gel for 3 h with a constant current of 20 mA per gel (38). After electrophoresis, the gel was allowed to equilibrate in transfer buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol, pH 8.3) at 20 C. Gels stained with Coomassie Blue were used to visualize proteins; unfixed gels were used for western blot analysis. The proteins were electrophoretically transferred overnight to nitrocellulose (30 V, 40 mA) in a Bio-Rad trans-blot unit (Bio-Rad Laboratories, Richmond, CA). The transfers were incubated for 1.5 h at 37 C with blocking buffer (1 part 10× PBS, 1 part fetal calf serum, 8 parts H_2O), then overnight with ascites (1:500 in blocking buffer) at room temperature with shaking. The blots were washed four times, 15 min per wash, with 1× PBS. Peroxidase-conjugated goat-anti-mouse IgG (Cappel Laboratories, Organon Teknika—Cappel, Duram, NC; 1:1,000 in blocking buffer) was added for 1.5 h with shaking. The blots were washed with shaking (four times, 15 min per wash) with 1× PBS and were then incubated with substrate (4-chloro-1-naphthol at 1 mg/ml in 5 ml of 0.075 M Tris-HCl, pH 7.5, containing 0.05 ml of 30% H_2O_2) at room temperature with shaking. After staining, the membranes were rinsed with distilled water and air-dried.

RESULTS

Indirect ELISA. Indirect ELISA was used to titer ascites and test its specificity against brown and gray isolates of *T. basicola* and other fungi commonly found in San Joaquin Valley cotton field soils. The fungi tested were *Rhizoctonia solani, Fusarium oxysporum* f. sp. *vasinfectum, V. dahliae, Sclerotinia sclerotiorum, S. minor, Trichoderma* sp., and *Penicillium* sp. The polyclonal ascites, obtained from mice immunized with both cytosol and cell wall soluble proteins, was specific to *T. basicola* with minimal cross-reactivity with the other fungi (Fig. 1). The ascites showed considerable reactivity with the brown isolates of *T. basicola* but was more specific to the gray, allowing these isolates to be separated serologically (Fig. 2). There were significant differences in ELISA rates between the brown and gray isolates (Student's t test, $P < 0.01$) at dilutions of ascites from 1:50 to 1:10,000.

Fungal capture sandwich ELISA. The sensitivity of fungal capture sandwich ELISA for *T. basicola* was tested. Known concentrations of *T. basicola* were serially diluted with blocking buffer. The ELISA dose response curves for *T. basicola* standards, containing 0.1 ng to 10 μg of protein, were sigmoidal (Fig. 3) and were fitted by iterative regression to the classic four-parameter logistic equation. As little as 1 ng of *T. basicola* (approximately one chlamydospore of the fungus as determined by dry-weight measurement, B. A. Holtz and A. R. Weinhold, *unpublished data*) was detected. The sensitivity of the fungal capture sandwich

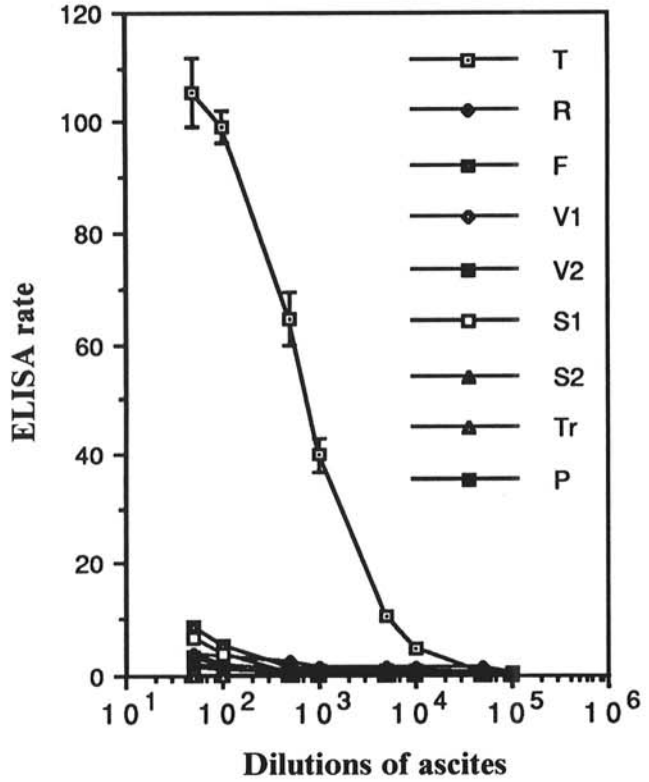

Fig. 1. Reactivity of ascites immunoglobulin G (IgG) raised against soluble cytosol and cell wall protein extracts of *Thielaviopsis basicola*, against soluble cytosol proteins of *T. basicola* (T), *Rhizoctonia solani* (R), *Fusarium oxysporum* f. sp. *vasinfectum* (F), *Verticillium dahliae* (V1, V2), *Sclerotinia sclerotiorum* (S1), *S. minor* (S2), *Trichoderma* sp. (Tr), and *Penicillium* sp. (P) in indirect enzyme-linked immunosorbent assay (ELISA). The experiment was repeated four times, twice with IgG raised against a cytosol immunogen, and twice with IgG raised against a cell wall immunogen. Error bars show the mean and standard error of three isolates (isolates 21, 22, and 26; Table 1) of *T. basicola*.

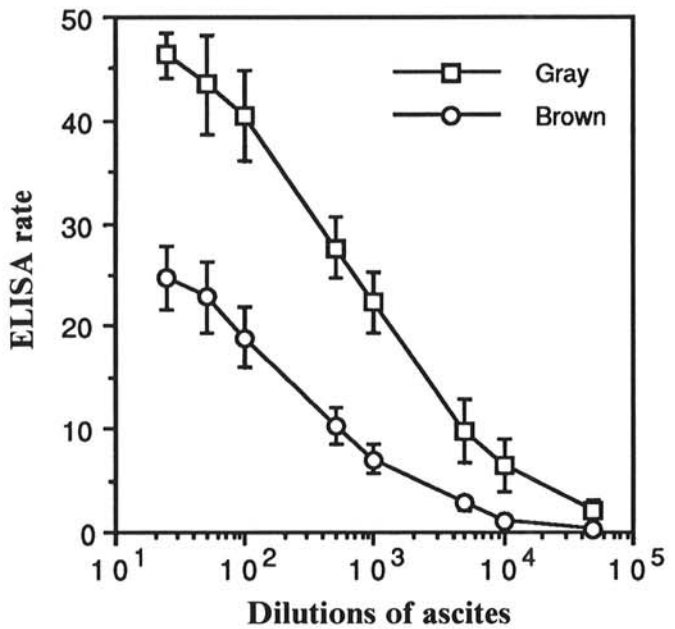

Fig. 2. Reactivity of *Thielaviopsis basicola*-specific immunoglobulin G raised against soluble cell wall proteins of isolate 26 (Table 1), with soluble cytosol proteins of brown (isolates 12, 14–16, and 19; Table 1) and gray (isolates 2, 5, 8, 9, 11, and 20; Table 1) isolates of *T. basicola* in indirect enzyme-linked immunosorbent assay (ELISA). Error bars show the mean and standard error of three replicates.

ELISA for *T. basicola* in cotton-root homogenate also was tested. *T. basicola* was serially diluted with healthy cotton-root homogenates and blocking buffer. Root tissue did not inhibit, interfere, or alter the sensitivity of the fungal capture sandwich ELISA for *T. basicola* (Fig. 3).

Fungal capture sandwich ELISA was used to detect *T. basicola* in diseased cotton seedlings exhibiting various levels of disease severity. Hypocotyls of 2-day-old cotton seedlings inoculated with *T. basicola* were removed 0, 2, 5, 8, 12, and 19 days after inoculation, and soluble protein extracts were prepared as described for the initial antigen. *T. basicola* could be detected in cotton roots as soon as 2 days after inoculation (Fig. 4). Fungal capture sandwich ELISA also was used to quantify *T. basicola* in these diseased cotton seedlings. ELISA rates of the diseased plants were compared to rates of *T. basicola* standards, and quantification was done by interpolation from the fitted standard curves by the four-parameter logistic equation (Table 2). An average of 3 ng of *T. basicola* protein could be quantified within roots as soon as 2 days after inoculation.

Immunofluorescence microscopy. Endoconidia and hyphae of *T. basicola* were fluorescent and easily distinguishable from the epidermal tissue of infected cotton plants (Fig. 5A). The hyphae and endoconidia were more strongly fluorescent than the thick-walled melanized chlamydospores. The cotton tissue was only slightly autofluorescent, except for some epidermal glands that were very autofluorescent. No fluorescence was observed in controls where ascites from an unimmunized mouse were used, the immunized ascites was omitted, or uninfected tissue was incubated with the ascites.

Western blot analysis. The soluble cytosol and cell wall protein fractions of *T. basicola* were separated by SDS polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose. The ascites, obtained from mice immunized with cell wall proteins, was tested for reactivity with the soluble cytosol

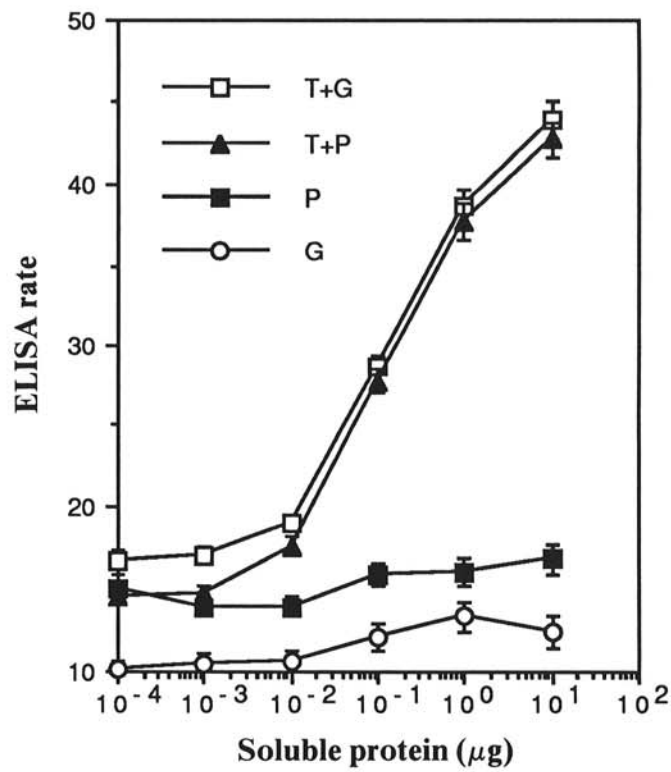

Fig. 3. The sensitivity of fungal capture sandwich enzyme-linked immunosorbent assay (ELISA) for *Thielaviopsis basicola* in cotton-root homogenates. *T. basicola* soluble protein standards (T) were serially diluted from 10 μg to 0.1 ng in both blocking buffer (phosphate buffered saline-Tween containing 0.1% gelatin) (G) and healthy cotton-root homogenate (P) and added to the plates, 0.1 ml per well. Error bars show the mean and standard error of three replicates.

and cell wall proteins on these blots. The ascites was reactive against a wide range of soluble proteins of *T. basicola*; some of which appeared to be in both cytosol and cell wall preparations (Fig. 6).

DISCUSSION

Several publications have reported various degrees of specificity of antisera produced against fungal antigens (1,9,24,27,30,37,41). Many of these antisera were prepared with tissue preparations containing fungal cell walls or cell wall materials. Gerik et al (17) stated that antigen preparations of these types could evoke antibodies to chitin or other cell wall polysaccharides common to most fungi. They obtained a more specific sera using only soluble fungal proteins as the antigen. In contrast, El-Nashaar et al (14) made polyclonal antisera to soluble hyphal antigens and to cell wall antigens. They found that the number of fungi that cross-reacted was reduced considerably when the cell wall fraction was used as an immunogen instead of using the soluble cytosol fraction.

In our study polyclonal antisera were produced to both a cell wall fraction and a soluble cytosol fraction of *T. basicola*. Both indirect ELISA and fungal capture sandwich ELISA revealed no difference in specificity of the cell wall and cytosol anitsera, and antibodies to each immunizing fraction reacted with the other fraction. Denaturing one-dimensional polyacrylamide gel electrophoresis and western blot analysis showed bands common to the cell wall and cytosol preparations. Immunofluorescence microscopy also revealed no difference in specificity of the cell wall and cytosol antisera for *T. basicola* on root tissue (B. A. Holtz and A. R. Weinhold, *unpublished data*). These results support the conclusion that there are antigens common to both immunogen preparations.

TABLE 2. Quantification of *Thielaviopsis basicola* in diseased cotton roots with fungal capture sandwich enzyme-linked immunosorbent assay (ELISA)

Days postinfection[a]	ELISA rate[b]	Total nanograms[c]	Nanograms per root[d]
0	16.44	0.00	0.00
2	23.16	35.17	3.52
8	26.07	68.78	6.88
12	29.82	144.28	14.43
19	31.75	209.08	20.91

[a]Cotton-seedling hypocotyls inoculated with *T. basicola* were sampled 0, 2, 8, 12, and 19 days after inoculation. Soluble cotton-root homogenates were prepared and fungal capture sandwich ELISA was used to detect *T. basicola*. The table shows the mean of three replicates.

[b]The rate of color development was monitored on a Multiskan ELISA reader interfaced with a Macintosh computer. The ELISA rates (ΔA_{405nm}/min $\times$ 10^3) were calculated by linear regression from three or more readings as the change in absorbance or color development above the blank from the time the substrate was initially added.

[c]The ELISA rates from the diseased root extracts were compared to ELISA rates from *T. basicola* standards. The *T. basicola* standards were sigmoidal and were fitted by iterative regression to a classic four-parameter logistic equation. Quantification of *T. basicola* in extracts of diseased cotton root were done by interpolation from the rearranged four-parameter logistic equation. The four parameters of the best fit of the standard curve in Figure 3 (lower asymptote: a_1 = 15.18 rate units; upper asymptote: a_2 = 44.36 rate units; midpoint of curve: a_3 = 0.146 ng; slope: a_4 = 0.73) were used in the equation to obtain the data in this table.

[d]There were 10 plants per treatment; average nanograms of *T. basicola* per root were calculated by dividing the total nanograms of *T. basicola* by 10.

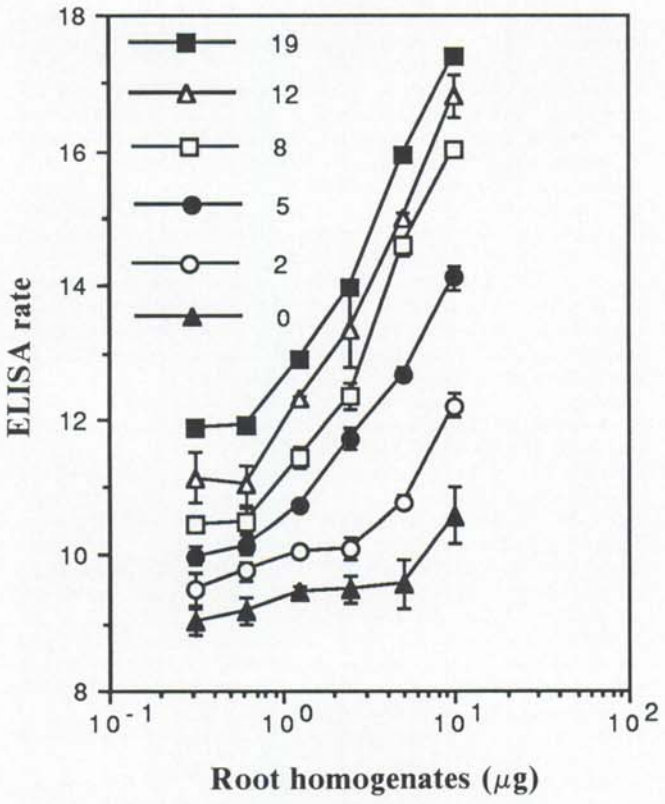

Fig. 4. Detection of *Thielaviopsis basicola* in diseased cotton seedlings by fungal capture sandwich enzyme-linked immunosorbent assay (ELISA). Hypocotyls of cotton seedlings inoculated with *T. basicola* were removed 0, 2, 5, 8, 12, and 19 days after inoculation, and soluble root homogenates were prepared as described for the initial immunogen. Error bars show the 2nd standard error of three replicates.

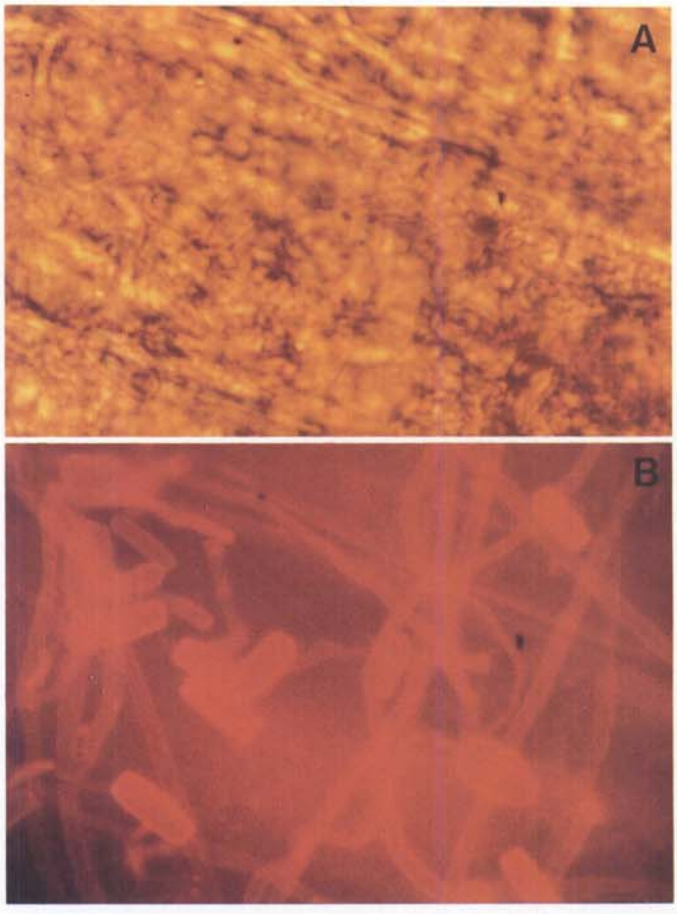

Fig. 5. Immunofluorescence microscopy of *Thielaviopsis basicola* endoconidia and hyphae on a diseased cotton root. The photographs show the same field of view (×750): **A,** under normal and **B,** UV light.

The polyclonal antisera were highly specific for *T. basicola* with minimal cross-reactivity with other soilborne fungi commonly found in San Joaquin Valley cotton fields. The antisera were not tested against any of the *Chalara*-like anamorphs, which are considered taxonomically related to *T. basicola*, because none were found in association with cotton roots.

T. basicola exists in nature in two distinct forms that have been called brown and gray cultural types (36). Prior to our work, the two forms could only be distinguished by their cultural characteristics on PDA (20,25) and by their respective pathogenicities (36). Both methods of identification are slow, subjective, and time-consuming. The antisera were reactive to both cultural types but were significantly more reactive with the gray cultural type. This was probably because the gray type was used initially as the immunogen. The ELISA we describe here provides another method by which the brown and gray cultural types can be distinguished.

A possible explanation for the differences between brown and gray cultural types is provided by Bottacin et al (4). They found different double-stranded ribonucleic acid (dsRNA) banding patterns in the two cultural types. These patterns could be due to mycoviruses or free dsRNAs. It is possible that these could alter *T. basicola*'s pathogenicity, its growth in culture, and ultimately the production of immunologically distinct proteins. This speculation also is supported by a previous report of virus-like particles containing dsRNA in cultures of *T. basicola* (5).

The fungal capture sandwich ELISA described here was able to accurately and reproducibly detect and quantify *T. basicola* in diseased root tissue. The sensitivity of the fungal capture sandwich ELISA was sufficient to allow the detection and quantification of *T. basicola* antigens in root extracts 2 days after infection.

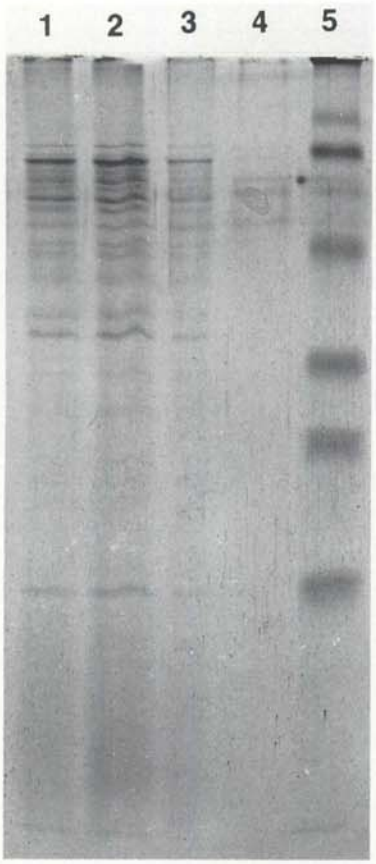

Fig. 6. Western blot analysis of cytosol (lanes 1 and 2) and cell wall (lanes 3 and 4) soluble proteins of *Thielaviopsis basicola* blotted onto nitrocellulose, incubated with *T. basicola*-specific immunoglobulin G (IgG), and stained with goat–anti-mouse IgG coupled to peroxidase. Protein standards (Bio-Rad) of known molecular masses ranging from 116 to 14 kDa are shown on the right (lane 5).

This ELISA should be usable for rapid field diagnosis and epidemiological studies of black root rot.

Other researchers reported similar results using DAS-ELISA to detect fungal antigens in plant tissue (7,14,29,37). DAS-ELISA usually requires raising IgG from two distinct animal sources. Our ELISA uses a single polyclonal IgG prepared from mouse ascites fluid. Unmodified IgG was used as the fungal trapping reagent. A portion of the same IgG was biotinylated and used as the detection antibody. Biotin is relatively polar and can be coupled to antibodies with a "spacer arm" eight to 14 carbons long under very mild conditions (31). Avidin may then be used as a stable, high-affinity second-step reagent that may be coupled with fluorochromes, enzymes, ferritin, or other molecules (2). The use of avidin as a sandwich reagent avoids the use of anti-immunoglobulin antibodies. The high sensitivity of the assay is due to the tight binding (binding coefficient $K_a = 10^{15}$ M^{-1}) of avidin to biotin (18).

For pilot serological studies with small amounts of fungal antigens, the production of polyclonal ascites in mice is an efficient alternative to the use and long-term maintenance of rabbits for antibody production. The ascites from three or four mice can yield amounts and titers of antibody comparable to a terminal bleed of an immunized rabbit (22). The methods developed here also could be extended to derive monoclonal antibodies that could give even better definitions of *T. basicola* serovars (isolates) and provide an "infinite" source of defined antibody.

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